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Perseverative responding and neuroanatomical alterations in adult heterozygous reeler mice are mitigated by neonatal estrogen administration

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Excitation-to-inhibition ratio

Summary According to the ''extreme-male brain'' theory, elevated fetal testosterone levels may partly explain the skewed sex ratio found in Autism Spectrum Disorders (ASD). Correcting this testosterone imbalance by increasing estrogen levels may mitigate the abnormal phenotype. Accordingly, while control heterozygous reeler (rl/+) male mice — a putative model of neuroanatomical and behavioral endophenotypes in ASD — show a decreased number of Purkinje cells (PC) compared to control wild-type (+/+) littermates, neonatal estradiol administration has been shown to correct this deficit in the short-term (i.e. on postnatal day 15). Here, we further investigated the neuroanatomical and behavioral abnormalities of rl/+ male mice and the potential compensatory effects of neonatal treatment with estradiol. In a longitudinal study, we observed that: i) infant rl/+ mice showed reduced motivation for social stimuli; ii) adult rl/+ male mice showed reduced cognitive flexibility; iii) the number of amygdalar parvalbumin-positive GABAergic interneurons were remarkably reduced in rl/+ mice; iv) neonatal estradiol administration into the cisterna magna reverted the abnormal profile both at the behavioral and at the neuroanatomical level in the amygdala but did not compensate for the cerebellar abnormalities in adulthood. This study supports the view that an increased excitation-to-inhibition ratio in the cerebellum and in the amygdala during a critical window of development could be crucial to the social and cognitive phenotype of male rl/+ mice, and that acute estradiol treatment during this critical window may mitigate symptoms' severity.

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1. Introduction

Autism spectrum disorder (ASD), a complex syndrome characterized by impaired theory of mind, increased perseveration and abnormal executive function (Goldstein et al., 2001; Pellicano, 2007), affects six per 1000 children with a 4:1 male-to-female ratio. Structural studies have revealed anatomical alterations in the frontal lobes, amygdala and cerebellum in ASD (reviewed by Amaral et al., 2008). Loss of cerebellar Purkinje cells (PCs) is the most consistent postmortem neuropathological finding in ASD (see Kemper and Bauman, 1998, for a review), but it is not clear how this neuropathological finding relates to the behavioral deficits in ASD. Relevant to this issue, recently published evidence supports the view that Purkinje cell loss in mice is associated with repetitive behavior (Martin et al., 2010).

In spite of well-defined behavioral scales describing the core symptoms of ASD, little is known about its specific etiology (Ronald et al., 2006). Although gene × environment environment interactions are likely to be involved in the etiology of ASD (Ronald et al., 2006; Laviola et al., 2009), the specific mediators and their precise interplay have remained elusive. Recently, based on the heavily male-biased prevalence of the disease, an 'extreme-male brain theory' has been proposed (Knickmeyer and Baron-Cohen, 2006). This theory suggests that elevated fetal testosterone levels favor the onset of ASD symptoms. Accordingly, whilst control females outperform males in nonverbal signs understanding, theory of mind capabilities and language-related skills, ASD patients perform poorly in the aforementioned specific domains. The extreme-male brain theory is further supported by recent genetic findings indicating a significant association between sex steroid genes (estrogen receptor β and aromatase, the enzyme converting testosterone to estradiol) and autistic traits, respectively Asperger Syndrome (Chakrabarti et al., 2009).

While it is unlikely that elevated fetal testosterone alone leads to ASD, excess fetal or perinatal testosterone may precipitate the phenotype of genetically vulnerable individuals. Among several putative genes, reelin has been proposed to represent a valid candidate in the pathophysiology of ASD (Persico et al., 2001; Keller and Persico, 2003; Fatemi et al., 2005; Eastwood and Harrison, 2006; Abrahams and Geschwind, 2008). Reelin, a large secreted protein critical for neuron positioning during brain development (D'Arcangelo et al., 1995; Tissir and Goffinet, 2003), is produced by Cajal-Retzius cells in the marginal zone of the developing cerebral cortex, and by cerebellar granule cells. Reelin binds to the very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2), both expressed by rodent and human PCs (Kim et al., 1996; Perez-Garcia et al., 2004). PCs of neonatal and adult rodents express aromatase (Biamonte et al., 2009), estrogen receptor β (Price and Handa, 2000; Jakab et al., 2001; Andreescu et al., 2007; Biamonte et al., 2009) as well as androgen receptor (Simerly et al., 1990). Estrogens promote the development and survival of PCs (Sakamoto et al., 2003) and PCs themselves are known to be a site of synthesis of estrogens (Tsutsui et al., 2000). Since PCs simultaneously express reelin receptors and receptors for neuroactive steroids, they could represent a common target of reelin and steroid signaling pathways (Biamonte et al., 2009).

Adult male heterozygous (rl/+) mice, haploinsufficient for reelin, display reduced numbers of PCs, in comparison to female rl/+ mice and wild-type (+/+) mice of either sex (Hadj-Sahraoui et al., 1996). Additionally, high testosterone/estradiol (T/E) ratios in the cerebellum are observed in rl/+ mice, and a single injection in the *cisterna magna* — on postnatal day 5 (P5) — of the estrogen receptor agonist 17β-Estradiol (17β-E) up-regulates reelin mRNA in the cerebellum, and reverses the PC loss in rl/+ mice at P15 (Biamonte et al., 2009). In the light of the extensive connections between the cerebellum and the neocortex (Strick et al., 2009), we also hypothesized the role of altered cerebro-cerebellar loops for behavioral and attentional deficits in rl/+ mice. To investigate the functional significance of this hypothesis, here we compared neonatal social capabilities (homing test, Bignami, 1996) and adult attentional set-shifting abilities in rl/+ and +/+ male mice exposed to 17β-E or vehicle on postnatal day 5. Based on these evidences and on the synergistic role played by the amygdala and the prefrontal cortex in regulating purposive behavior (reviewed in Barbas, 2000; Bechara et al., 2000; Birrell and Brown, 2000) we also measured the number of Parvalbumin-positive, GABAergic inhibitory interneurons in the prefrontal cortex and amygdala, and of PCs in the cerebellum. Finally, we assessed whether neonatal estradiol normalized the abnormal phenotype we predicted to observe in adult mice.

2. Materials and methods

2.1. Subjects

B6C3Fe heterozygous female and wild-type male mice, originally purchased from Jackson Laboratories (USA) were bred in our laboratory. Two females were housed with one male in 33 cm × 13 cm × 14 cm Plexiglas boxes. After ca. 2 weeks, the male was removed and the females were housed individually and daily checked for delivery. Food (Enriched standard diet purchased from Mucedola, Settimo Milanese, Italy) and water were continuously available. Mice were housed in a temperature-controlled room at 21 ± 1 °C, (relative humidity 60 ± 10%) under a reverse 12:12 h light—dark cycle, with lights off at 7:00 a.m. All animal handling and experimental procedures were performed according to European Communities guidelines (EC Council Directive 86/609), Italian legislation on animal experimentation (Decreto L.vo 116/92) and NIH guide for the care and use of laboratory animals.

Only male offspring were used in the study. Experimenters were blind to subject genotype and treatment during both behavioral testing and neuroanatomical analyses. A first group of subjects were tested for ultrasound vocalizations (P7), grasping reflex (P7 and P11), homing (P9), social preference (P150), and attentional set-shifting (P180) and body-weight gain at P 5, 7, 11, 21, 80, 90, 100, 110, 120, 130, 140, 150. A second — independent — group of subjects were assessed for rotarod (P70), fear conditioning (P150) and for immunohistochemistry (P180). For each treatment group (17β-Estradiol or vehicle) 10 dams per replicate were mated. This resulted in an average of 7 dams giving birth per group per replicate. The offspring were then used for treatment. In order to avoid potential litter effects, no more than two littermates of the same genotype were used in each experi-

ment. This breeding strategy resulted in an average of 8–9 individuals per group in each test. Details on the specific number of subjects used are reported in the figure legends. In the present study we aimed at addressing neonatal sensorimotor development and adult cognitive responses in estradiol-treated rl/+ mice. Based on this, the behavioral tests were performed at times – within the target ages (i.e. infancy and adulthood) – that maximized the possibility to observe variation in the experiments. Therefore, neonatal vocalizations were measured when they reach their peak; homing performance was addressed when eyes are still closed but locomotion is present; grasping was measured twice in order to observe an immature response (P7) and an almost fully mature response (P11). Conversely, adult tests were performed through a schedule that minimized carry-over effects due to test battery and maximized the information obtained by a single individual. Thus, in adulthood, the test characterized by the lowest invasiveness was performed first and the other was performed after a long period of time.

2.2. In vivo estradiol treatment

On postnatal day 5 (P5), mice were treated with the estrogen receptor agonist 17β-Estradiol (purchased from Sigma–Aldrich, Milano, Italy), dissolved in DMSO (final concentration 5 mg/ml). Neonates were anaesthetized on crushed ice for 3 min and were then injected with a single dose of 1 μl using a Hamilton μl 7000 syringe (Aldrich Chemical Company, Inc., USA) into the *cisterna magna*, as published previously (Biamonte et al., 2009). Control experiments were performed by injecting animals with vehicle (Veh) only. Mice were genotyped for the rl^{jx} mutation and sex (Sry gene) as described in (Biamonte et al., 2009). The animals were sacrificed around seven months of age and the brain was sampled for determination of Purkinje Cell (PC) number in the cerebellum, and numbers of parvalbumin-positive in amygdala and prefrontal cortex, using stereological methods (see below).

2.3. Ultrasound vocalizations

Ultrasound emission in newborn rodents in response to separation from the mother is considered a reliable measure of neurobehavioral development. Male rl/+ and +/+ mice exposed to vehicle or 17β-E were assessed for ultrasonic vocalizations in a 2-min test on P7. Ultrasonic calls were recorded in a sound-attenuating chamber (Amplisilence, I-10070 Robassomero, Italy) during the dark period of the L/D cycle, between 11 a.m. and 3 p.m. Pups were removed from the litter and individually placed in a double wall glass container (diameter, 5 cm; height, 10 cm). Two openings in the external glass wall allowed water to be continuously pumped from a water bath into the double wall and, ultimately, to return to the pump. This maintained the inner surface of the container at the same temperature (26 ± 1 °C). The number of ultrasonic calls emitted during the 2-min test was assessed by recording and then scoring the audible frequencies output of a S-25 Bat Detector (Ultra-Sound Advice, London, UK) tuned at ±40 kHz range centered on 60 kHz, according to the procedure described by Branchi et al. (2001).

2.4. Grasping reflex

In response to a tactile stimulation of forepaw palm the pup grasps the object, a reflex also observed in the human newborn. To evaluate the strength of grasping on P9 and P11, pups were placed on a narrow mesh metal grid in a horizontal position (0°). The grid was then progressively tilted to a vertical position (+90°) and further to a horizontal upside-down position (180°). Because of its own weight and rotation of the grasped wire, the pup will eventually fall from the grid onto a soft paper bedding. The angle of the grid at which the pup falls is recorded as "fall angle". The test was repeated three times, and the largest angle reached was recorded (Laviola et al., 2006).

2.5. Homing test

In order to evaluate neonatal preference for the nest-cage odor, as an indicator of the individual capabilities to recognize maternal olfactory stimuli, we performed a homing test. Specifically, on P9, pups were isolated in a clean cage and kept for 30 min at a temperature of 28 ± 1 °C. Subjects were then transferred individually into a Plexiglas arena (36 cm × 22.5 cm, walls 10 cm high) with a grid-floor subdivided by white lines in 6 quadrants. Wood shavings from the home-cage were evenly spread under the wire-mesh floor on one side of the arena (14 cm × 22.5 cm, goal area) and the pup was placed close to the wall on the opposite side. The latency to put both forelimbs on the goal area was recorded (cut-off time 5 min). In addition, pup's overall activity was measured by counting the number of squares entered during the 5-min test period (Laviola et al., 2006). Since not all the subjects completed the task within the cut-off time, we also measured the percentage of pups reaching the goal area within the 5-min test session.

2.6. Social preference test

In order to quantify levels of sociability in mice, we performed a social interaction test, evaluating the preference for a novel unfamiliar subject over a familiar one (Moy et al., 2004). The apparatus (see Fig. 1 for a sketch) consisted of an opaque polycarbonate box (two lateral compartments of 30 cm × 35 cm × 30 cm connected by means of a central

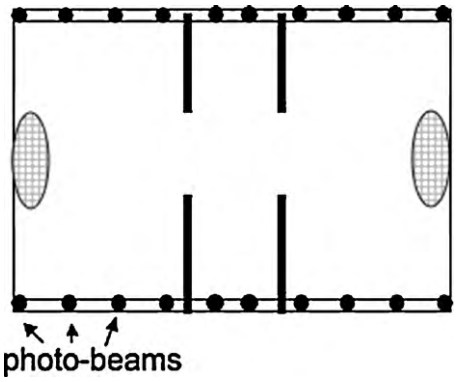

Figure 1 Sketch of the apparatus used for the social interaction task.

compartment of 10 cm × 35 cm × 30 cm) with removable partitions subdividing the box into three chambers. The partitions had openings (20 cm height) that allowed the animal to move freely from one chamber to another. Two infrared photo-beams (positioned at 25 mm from the floor) allowed the detection of subject's locomotor activity. The time spent in each chamber and the transitions between each chamber were recorded by a computer with custom-made software. The apparatus was positioned such as to minimize gradients in light, temperature, sound and other environmental conditions that could produce a side preference.

Metal wheel-shaped boxes (19.5 cm diameter) were positioned into each of the two side chambers of the apparatus. These boxes had wire-mesh grid walls with thin, widely spaced bars that allowed nose contact between the bars, but prevented more complex social contact, including the possibility of aggressive interactions. The boxes and the apparatus were thoroughly cleaned with a mixture of water and ethanol and dried with paper towels after each subject. The procedure consisted of three trials:

TASK 1: The experimental animal was placed in the central compartment and allowed to freely explore the entire apparatus for 15 min. This session was aimed at evaluating whether subjects of the different groups showed differences in basal locomotor activity levels; additionally, this session served the aim to assess the presence of any bias preference for one of the two side chambers of the apparatus. The subject was returned to the central compartment at the end of the habituation session.

TASK 2: Immediately before this trial, an unfamiliar same-sex, same-age adult C57BL/6J mouse (the stimulus subject) was put inside one of the two wheels positioned in each of the two side chambers of the apparatus. The stimulus subject was able to move inside the wheel-shaped box, but not to escape it. The second wheel, positioned in the opposite chamber was left empty. The experimental mouse was allowed to move freely throughout all three chambers of the apparatus for a 15-min test session. The location of the stimulus mouse and of the empty wheel-shaped box was randomized between left and right chambers, to avoid any biased chamber preference. Measures were taken of entries and time spent into each of the two end-chambers, namely the social side containing the unfamiliar mouse in the box, and the empty side containing the empty box only.

TASK 3: For this trial, a novel unfamiliar mouse was placed into the box that was empty in the previous trial, and that was now denominated unfamiliar. The experimental subject was then allowed to freely explore the apparatus for an additional 15-min test session. During this session, the number of crossings and the time spent in both the (now) familiar and unfamiliar sides were measured.

Compared to other sociability tasks, the sequential procedure adopted in this study — based on previous work done in the Crawley's group (see e.g. Crawley, 2007) — specifically allows addressing social approach during task 2 (comparison between the empty side and the novel stimulus) and social recognition during task 3 (comparison between the familiar and the unfamiliar stimuli). Task 3 is also associated with a social memory component. These aspects have been extensively discussed by Crawley (2004).

2.7. Attentional set-shifting test

We adopted the attentional set-shifting task, originally developed by Birrell and Brown (2000) and modified by Colacicco et al. (2002) (see also Macri et al., 2009).

Apparatus: the apparatus, identical to the one used by Colacicco et al. (2002), was an opaque PVC U-shaped box with a grid-floor and a transparent plexiglass lid (45 cm × 30 cm × 15 cm). Two identical choice compartments (15 cm × 15 cm) at one end of the apparatus could be accessed through sliding doors from a starting compartment (30 cm × 30 cm). A cylindrical food cup (40 mm diameter, 35 mm high) in each choice compartment could be baited with a small piece of cereal (30 mg; Honey Nut Loop, Kellogg). The food was then covered with a layer of scented digging medium (20 mm, see table). The presence or absence of food reward in a cup was indicated by either tactile (type of digging medium) or olfactory stimuli (scent of the digging medium).

Habituation: on the day before testing, mice were given access to the apparatus for 30 min. Following this preliminary exposure mice were trained to dig into food-baited bowls during a series of 9 consecutive trials. During the first three trials mice were allowed to explore the apparatus until two food rewards, both located on the surface of the empty bowls, were retrieved. Between trials 3—6, food rewards were located on the surface of the digging media inside the bowls and mice were allowed to explore the apparatus until retrieval. Between trials 6—9, food rewards were located underneath the digging media. This procedure allowed mice to perform reliable digging.

Testing: we used the same experimental protocol adopted by Garner et al. (2006). Briefly, a trial was initiated by raising the sliding wall to give the mouse access to the two digging bowls, only one of which was baited. Food-restricted mice (85% of their original body weight at the beginning of the experiment) were required to dig into a rewarded (food-baited) bowl to obtain highly palatable food pellets. Digging bowls varied across two dimensions (digging medium and scent). Digging media and odors exemplars used in this study are reported in Table 1. During simple discrimination (SD) mice had to learn to discriminate between two different odors or digging media. After this stage, mice were required to perform a compound discrimination (CD), during which the baited stimulus of the previous stage was presented together with another, newly introduced, irrelevant stimulus of the other dimension. Despite the presence of the new stimulus, the correct and incorrect exemplars remained constant (e.g. cinnamon odor was rewarded when presented in combination with either sawdust or foam paper while thyme was not rewarded independently of the digging medium, see Table 1). At the end of this stage mice had to perform CD

Table 1 Stimulus exemplars used in the task.

Dimension	Pairing (exemplar 1)	Pairing (exemplar 2)
Odor	Cinnamon—Thyme	Anise—Thyme
Medium	Sawdust—Cotton	Sawdust—PaperChip

Compound discriminations were based on fixed combinations of pairs of exemplars. The sequences of these combinations were presented in random order.

reversal learning (CDR). For the reversal, the exemplars and the relevant dimension were unchanged: the mouse had to learn that the previously correct stimulus was now incorrect. Stimulus presentation and counterbalancing was identical to the one used by Garner et al. (2006). A stage was considered complete when the mouse achieved a criterion of 8 correct trials out of 10 (Garner et al., 2006). A session would continue until the animal ceased responding. Normally, mice would give a good profile of responses for about one hour, time after which they would just ignore the reward. Since the end of a session depended on the individual motivation to perform the task, subjects performed a variable number of trials each day. However, one hour of responding resulted in an average of approximately 30 trials per day per subject.

2.8. Fear conditioning

Subjects were tested for tone and context fear-conditioning test on P65. All the procedures were performed during the light cycle between 10 a.m. and 4 p.m. in a soundproof room.

Apparatus: A conditioning chamber (Coulbourn Instruments, Allentown, USA), was used for training session. Side-walls of the chamber were made of grey Plexiglas, while ceiling was made of transparent Plexiglas to allow video-recording. The grid-floor of the chamber (steels spaced by 1.5 cm) was connected to a shock generator scrambler. Context testing took place in the same conditioning chamber, while tone testing took place in a white Plexiglas box (21 cm $\times$ 18 cm $\times$ 45 cm) with black stripes applied on the walls. The chamber was cleaned with an odorless ethanol 50% solution before the placement of each animal into the chamber during conditioning, tone and context testing sessions.

Procedure: Following a 120 s of acclimation to the conditioning apparatus (baseline), three trials were delivered consisting in presentation of a 2 kHz, 90 dB tone of 30 s duration (CS). The last 2 s of each presented tone were paired with a 1 mA foot-shock (US). Each tone was separated from the following by a 60 s inter-trial interval (ITI) without presentation of either tone or shock. After 24 h all the animals were subjected to both the context and tone testing in the same experimental room where training session was performed. Using the same random order of the training procedure, subjects were placed in the original training context for 240 s of observation (context test). Then, the tone test was performed in the new context (see Apparatus description). After 120 s of acclimation, animals received 120 s tone similar to the conditioning session but shock free (tone test).

Spontaneous behavior was recorded for each mouse during training, context and tone testing and behavioral responses during tests were scored by means of the software ''The Observer'' (Noldus, Wageningen 6700 AG, The Netherlands). During each phase (training, context and tone tests), frequency and duration of the following behavioral items were collected:

- locomotion: crossing of three equally sized virtual sectors;
- *rearing*: head raising and standing on hind legs; standing on hind legs;
- *Sniffing*: self-explanatory;
- *grooming*: self-cleaning;
- *freezing*: behavioral immobility, except for respiration movements;
- *inactivity*: similar to *freezing* but small movements of head, ears or vibrissae are detectable.

2.9. Rotarod test

Rotarod apparatus (Basile, Comerio, Italy) was used to measure fore- and hind-limb motor coordination and balance. On the day of testing, mice were trained on the apparatus for at least three consecutive trials in which the rod was kept at constant speed (one trial at 0 rpm and two trials at 4 rpm). Once the trained animals were able to stay on the rotating rod at 4 rpm for 60 s, they proceeded to the test. Mice were placed individually for four consecutive trials (30-min inter-trial intervals) on the rod rotating at an accelerating speed from 4 to 40 (8, 16, 24, 32, 40) rpm in 300 s. The latency to fall off the rod and the maximal speed reached were automatically recorded (Marazziti et al., 2004).

2.10. Immunohistochemistry, brain tissue collection and processing

Adult animals were deeply anaesthetized by intraperitoneal injection of a mixture of 2 mg/ml ketamine, 0.2 ml/10 g body weight (Ketavet, Gellini farmaceutici, Italy) and 0.23 mg/ml medetomidine, 0.24 ml/10 g body weight (Domitor, Orion, Espoo, Finland). The animals were transcardially perfused with saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The brain was removed and placed in a cryoprotecting solution, frozen and stored at $-80\,^{\circ}$C until processing. Coronal sections were cut in a cryostat (Leitz) at 50 μm. Parvalbumin-positive (PV+) interneurons in the amygdala and prefrontal cortex were detected immunohistochemically by a modification of the avidin-biotin peroxidase method (using ABC kit, Vectastain). In brief, after washing in phosphate buffered saline (PBS), sections were soaked with bovine serum albumin in PBS for 1 h to block non-specific tissue antigen, and then incubated with a mouse anti-parvalbumin monoclonal antibody (SWANT, Bellinzona, Switzerland; 1:10,000 dilution in PBS). After an overnight incubation at $4\,^{\circ}$C, sections were washed with PBS, then incubated with secondary biotinylated anti-mouse antibody (Chemicon, USA; 1:200 dilution in PBS) for 1 h at room temperature. After washing with PBS, the sections were incubated with avidin DH-biotin peroxidase complex (dilution 1:100 in PBS) for 30 min at room temperature. Following a wash with PBS, immunoreactive products were visualized by 15 min incubation with 0.05% 3,3-diaminobenzidine and 0.01% hydrogen peroxide dissolved in 0.1 M phosphate buffer at room temperature.

Coronal cerebellum sections were processed and stained with Giemsa as previously described (Biamonte et al., 2009).

2.11. Stereological analysis

The optical fractionator method stereological design (West et al., 1991) was used to obtain unbiased estimates of total number of PV+ cells in the amygdala, prefrontal cortex, and of PCs in the cerebellum, using the Stereo Investigator system

(Stereo Investigator software, Version 4.04© 2000, MicroBrightField Europe, Magdeburg, Germany). A stack of MAC 5000controller modules (Ludl Electronic Products, Ltd., Hawthorne, NY, USA) was configured to interface an Olympus BX 50 microscope with a motorized stage and a HV-C20 Hitachi color digital camera with a Pentium II PC workstation. A three-dimensional optical dissector counting probe (*x, y, z* dimension of 30 μm $\times$ 30 μm $\times$ 10 μm respectively) was applied to a systematic random sample of sites of region of interest (ROI). For counting of amygdala PV+ neurons, coronal sections were taken through the amygdala along the rostro—caudal axis corresponding to approximately +0.70 mm to −2.46 mm relative to bregma in the mouse brain atlas of Paxinos and Franklin (2001). The right and left amygdala were counted separately. For counting of PV+ neurons in prefrontal cortex, coronal sections were collected through the rostro—caudal axis corresponding to approximately +3.20 mm to +2.10 mm relative to bregma (Paxinos and Franklin, 2001). The prefrontal cortex was further subdivided into four sub-regions: dorsolateral, lateral, ventral, and mediolateral. The left and right prefrontal cortex were counted separately. Counts of PCs were obtained as described previously (Biamonte et al., 2009).

2.12. Statistical analysis

For the analysis of ultrasound vocalizations, homing and social interaction paradigms, data were analyzed by two-way ANOVA, using Stat View software (version 4.0). The general model was a 2 genotype (+/+ and rl/+) $\times$ 2 treatment (17β-E and Vehicle). For the analysis of social interaction, the model was a repeated measures ANOVA for split-plot design with between subject and within subject factors: specifically the model was a 2 genotype (+/+ and rl/+) $\times$ 2 treatment (17β-E and Vehicle) $\times$ 2 side (left and right in task 1; social and empty in task 2; familiar and unfamiliar in task 3) $\times$ 3 time interval (5-min bins). For the analysis of PV+ neurons in the left and right prefrontal cortex the model was a 2 genotype (+/+ and rl/+) $\times$ 2 treatment (17β-E and Vehicle) $\times$ 4 sub-regions (dorsolateral, lateral, ventral, and mediolateral). For the analysis of PV+ neurons in the amygdala the model was a 2 genotype (+/+ and rl/+) $\times$ 2 treatment (17β-E and Vehicle) $\times$ 2 side (right and left). Genotype and treatment were between subject factors while all the others were within subject factors. Post hoc comparisons were performed when allowed (Tukey test) and significance level was set at $p < 0.05$. Attentional set-shifting: although presentation of odors and digging media was counterbalanced across groups, the two different dimensions might have elicited different responses if one dimension were more relevant than the other. For this reason data were transformed into *Z*-standard values ($Z_i = (R_i - M)/\partial$) for statistical analyses. However, for the sake of clarity, data are reported as original, non-transformed, values. The number of pups reaching the nest area during the homing test was analyzed through χ^2 test.

3. Results

3.1. Body-weight gain

As expected, body weight increased with age (age, $F(11,209) = 1946.4$, $p < 0.001$). Additionally, neonatal estradiol administration significantly reduced body weight in the offspring (treatment, $F(1,19) = 9.852$, $p < 0.05$). Thus, 17β-E-treated mice showed lower body weight than vehicle-treated controls. Yet, this effect varied depending on genotype and developmental stage. Specifically, while +/+ 17β-E-treated subjects consistently showed reduced body weight compared to controls, rl/+ 17β-E-treated mice showed reduced body weight in adulthood but not at weaning (treatment $\times$ genotype $\times$ day, $F(3,78) = 4.67$, $p < 0.01$, see Fig. 2).

3.2. Ultrasound vocalizations (UVs)

+/+ and rl/+ subjects exposed to vehicle (frequency/min 5.8 $\pm$ 2.3 and 6.0 $\pm$ 1.9 respectively, $N = 9$) or 17β-E (13.1 $\pm$ 3.7 and 9.5 $\pm$ 3.3 respectively, $N = 9$) showed indistinguishable levels of UVs emission on P7 (treatment $\times$ genotype, $F(1,31) = 0.2$; NS).

3.3. Grasping test

As expected, the *grasping reflex* improved between P7 and P11, with P11 subjects showing a larger falling angle compared to P7 mice (171.5 $\pm$ 1.4° vs. 130.4 $\pm$ 2.4°) (day, $F(1,27) = 105.740$; $p < 0.001$). However, neither genotype,

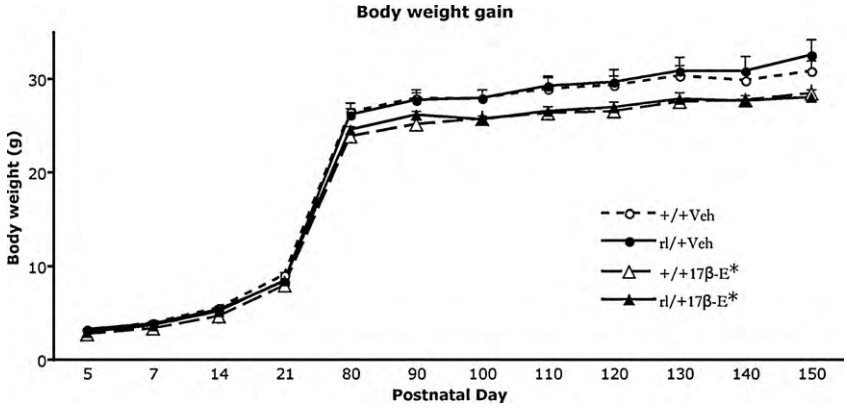

Figure 2 Body-weight gain in Veh-+/+ ($N = 8$), Veh-rl/+ ($N = 8$), 17β-E-+/+ ($N = 8$) and 17β-E-rl/+ ($N = 9$) subjects from infancy P5 through adulthood, P150. $^{*}p < 0.05$ significantly different from Veh-injected subjects.

Table 2 Homing test.

Neonatal injection	Genotype	
	+/+	rl/+
Veh	33	19.3*
17β-E	19.4*	37.5

Percentage of mice reaching the goal area in the homing test. *$P < 0.05$, significantly different from Veh-+/+ subjects ($N = 9$).

nor treatment modified the performance in the grasping reflex test on P7 and P11 (genotype × treatment, $F(1,27) = 0.071$, NS, and $F(1,27) = 0.576$, NS, respectively, $N = 8$). Taken together, the results of UVs and grasping test indicate the absence of gross neurobehavioral deficits in rl/+ mice.

3.4. Homing test

+/+ and rl/+ subjects exposed to vehicle and 17β-E showed similar levels of general locomotion, measured in terms of crossings (treatment × genotype, $F(1,31) = 0.863$; NS, data not shown). Not all subjects reached the goal area during the test session. We therefore analyzed the percentage of subjects reaching the goal area. The latter varied depending on genotype and treatment ($\chi^2 = 9.24$, $P < 0.01$). Specifically, whereas a significantly lower number of control rl/+ mice reached the goal area compared to control +/+ subjects, this difference was abolished by 17β-E treatment in rl/+ mice. Conversely, 17β-E treatment reduced the number of subjects reaching the goal area in +/+ mice (see Table 2). Since most of the subjects failed to reach the goal area during the homing task, these data did not meet the normality distribution assumption. Therefore we did not perform an ANOVA on latency. However, for the sake of completeness we report the average latency to reach the goal area by +/+ and rl/+ subjects exposed to vehicle (254.4 s and 220.5 s respectively) and 17β-E (209.1 s and 161.5 s respectively).

3.5. Social interaction

Task 1: General locomotion measured during the 10-min habituation to the test apparatus was not affected by genotype or treatment (genotype × treatment, $F(1,24) = 1.425$ NS, see Table 3).

Task 2: When mice were allowed to freely spend time in the empty compartment or in a compartment in which a conspecific was present, all mice spent a significantly higher proportion of time in the proximity of the social stimulus (side, $F(2,48) = 3.252$, $p < 0.05$). However, this parameter did not differ between groups (genotype × treatment, $F(1,24) = 3.747$, NS, see Table 3).

Task 3: When mice were faced with a choice between two social stimuli differing for familiarity, subjects did not show any preference for the familiar or the unfamiliar stimulus (side, $F(1,24) = 0.796$, NS). Additionally, in the absence of baseline differences between +/+ and rl/+ mice in preference for the unfamiliar social stimulus, neonatal treatment with 17β-E increased such preference in +/+ individuals (genotype × treatment, $F(1,24) = 4.319$, $P < 0.05$). Thus, 17β-E +/+ mice spent an increased amount of time in proximity of the unfamiliar stimulus compared to +/+-veh controls (see Table 3).

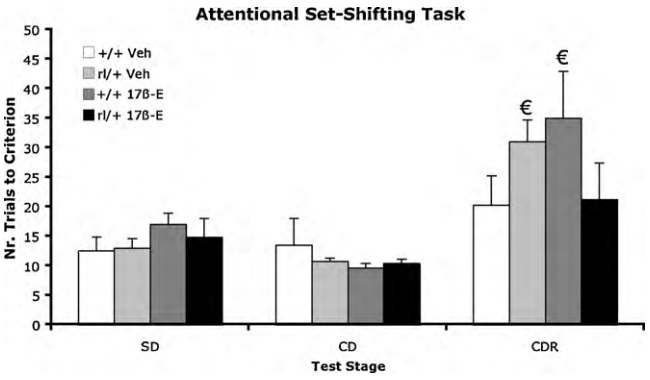

Figure 3 Mean number (±SEM) of trials required by Veh-+/+, Veh-rl/+, 17β-E-+/+ and 17β-E-rl/+ adult mice to complete the criterion (8/10) in each stage: SD = simple discrimination; CD = compound discrimination; CDR = compound discrimination reversal. € $p < 0.05$ significantly different from Veh-+/+ and 17β-E-rl/+ subjects using transformed scores ($N = 5$).

3.6. Attentional set-shifting test

The first two stages of the task require a simple discrimination and a perseveration in the same responding despite the presence of a distracter (CD). Therefore, mice are generally expected to learn SD and CD sooner than CDR, which involves the formation of a new rule. Accordingly, mice from all groups required a smaller number of trials to learn SD and CD compared to CDR. Additionally, we observed that mice showed a faster acquisition of the learning criterion when required to discriminate between digging media rather than odors (stimulus dimension $F(1,18) = 9.3$, $P < 0.01$).

Neither genotype nor treatment affected the SD and CD tasks (genotype × treatment: $F(1,16) = 0.4$, NS; $F_{1,16} = 0.3$, NS respectively). Conversely, as shown in Fig. 3, a differential

Table 3 Social interaction task.

	Veh +/+	Veh rl/+	17β-E +/+	17β-E rl/+
Task 1 (locomotion: no. of beam interruptions/s)	5.4 ± 0.3	6.0 ± 1.5	6.0 ± 1.2	20.7 ± 6.3
Task 2 (time spent in empty side, s)	343.8 ± 41.1	340.5 ± 73.2	443.7 ± 41.1	392.4 ± 73.2
Task 2 (time spent with social stimulus, s)	449.4 ± 41.7	497.7 ± 75.0	376.5 ± 38.1	418.5 ± 43.5
Task 3 (time spent with familiar stimulus, s)	447.3 ± 55.2	405.6 ± 66.3	252.9 ± 42.3	456.6 ± 21.9
Task 3 (time spent with unfamiliar stimulus, s)	348.6 ± 53.7	388.8 ± 63.6	576.3 ± 52.5*	341.1 ± 21.3

Relevant parameters in each task of the social interaction test ($n = 8$). *Significantly different from Veh +/+, $p < 0.05$.

Table 4 Fear conditioning.

Cue phase	Before tone onset				During tone presentation			
	Veh +/+	Veh rl/+	17β-E +/+	17β-E rl/+	Veh +/+	Veh rl/+	17β-E +/+	17β-E rl/+
Grooming (duration, s)	10.3 ± 3.9	4.4 ± 1.5	12.5 ± 2.2	6.2 ± 1.6	1.2 ± 1.2	2.35 ± 1.4	3.51 ± 1.5	4.16 ± 1.9
Rearing (frequency)	12.1 ± 1.9	15.5 ± 0.9	18.3 ± 0.8	15.6 ± 1.4	10.8 ± 2.3	9.5 ± 2.5	9.29 ± 1.6	7.4 ± 2.9
Crossing (frequency)	37.7 ± 10.1	53.7 ± 9.5	18.3 ± 2.9	43.4 ± 6.9	26.9 ± 8.3	17.8 ± 6.9	20.1 ± 2.8	14 ± 2.4
Freezing (duration, s)	0.2 ± 0.2	0	0	0	10.6 ± 4.0	20.9 ± 10.3	19.4 ± 5.0	23.6 ± 9.2
Immobility (duration, s)	5.0 ± 2.0	0	0.9 ± 0.9	3.7 ± 2.1	16.0 ± 6.6	18.5 ± 7.0	18.4 ± 7.0	29.1 ± 8.3

Behavioral parameters observed during the cue phase of the fear-conditioning test in Veh-+/+ ($N = 6$), Veh-rl/+ ($N = 6$), 17β-E-+/+ ($N = 6$) and 17β-E-rl/+ ($N = 6$).

response in the CDR task (genotype × treatment: $F(1,16) = 5.1$, $P < 0.05$) appeared as a function of genotype and early hormonal treatment. In particular, vehicle-treated +/+ mice learned CDR significantly quicker than rl/+ controls. Additionally, 17β-E neonatal treatment improved significantly the performance of rl/+ subjects, which showed an indistinguishable CDR learning compared to Veh +/+ subjects. Finally, as indicated by the fact that +/+ 17β-E-treated mice required a higher number of trials to learn CDR compared to Veh +/+ subjects, 17β-E treatment seemed to negatively affect attentional set-shifting *per se* in +/+ controls (see Fig. 3).

3.7. Fear conditioning

During the context phase all subjects spent approximately 3% of their time in freezing behavior and 5% inactive. However, apart from a small reduction in self-grooming observed in +/+ vehicle-treated subjects compared to the other groups, subjects of the four groups showed similar behavioral time budgeting (data not shown). During the baseline 3-min period of the cue phase (cue absent), subjects showed neither freezing nor inactive behavior (see Table 4). Conversely, following tone presentation all subjects showed a significant, though moderate, increase in both freezing (phase test, $F(1,19) = 30.14$, $p < 0.0001$) and inactivity (phase test, $F(1,19) = 26.71$, $p < 0.0001$). This difference also resulted in differential exhibition of the other behavioral patterns scored. However, although the cue presentation produced the expected fear-conditioning response (increased freezing and immobility), this response was similar in all experimental groups (genotype × treatment, freezing $F(1,19) = 0.202$, NS, and immobility $F(1,19) = 0.300$, NS, respectively).

3.8. Rotarod

While most of the subjects managed to keep balanced on the rotating rod at slow speed, increasing the number of revolutions per minute increased the frequency of falling (trials, $F(3,84) = 7.485$, $p < 0.05$). However, subjects of the different groups did not differ with respect to absolute falling latency (genotype × treatment, $F(1,28) = 0.332$, NS; Veh +/+ 204.44 ± 13.2, Veh rl/+ 240.25 ± 13.30, estr +/+ 215.09 ± 13.17, estr rl/+ 222.90 ± 12.84, $N = 9$).

3.9. Stereological analyses

Cerebellum: adult rl/+ mice showed a significantly lower number of PCs compared to +/+ mice, irrespectively of 17β-E treatment (genotype, $F(1,15) = 142.6$, $p < 0.01$, see Fig. 4).

Prefrontal cortex: neither genotype ($F(1,13) = 0.7$, NS) nor treatment ($F(1,13) = 1.5$, NS) or an interaction of both (treatment × genotype, $F(1,13) = 0.9$, NS) affected the number of GABAergic interneurons in the right prefrontal cortex (see Fig. 5). Identical results were obtained in the left prefrontal cortex (data not shown).

Amygdala: control rl/+ mice showed a decreased number of PV+ GABAergic interneurons compared to +/+ control mice (genotype, $F(1,12) = 13.0$, $p < 0.01$; $p < 0.05$ in post hoc comparisons between +/+-veh vs. rl/+-veh and +/+-17β-E vs. rl/+-17β-E mice). Additionally, 17β-E treatment

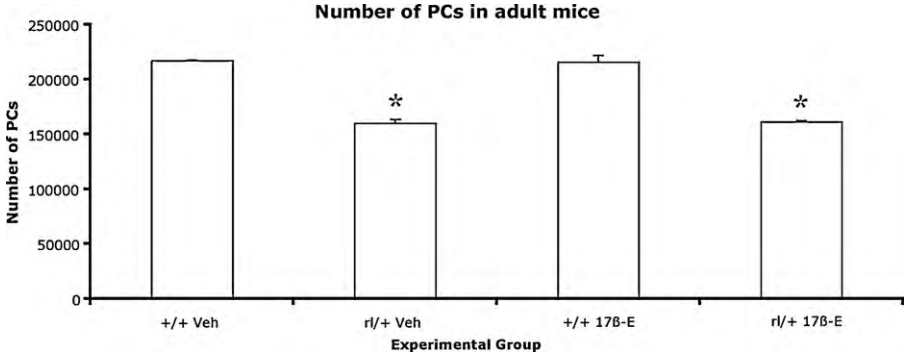

Figure 4 Stereological counts of Purkinje cells. Total number of PCs in vehicle-treated and 17β-E-treated +/+ and rl/+ mice at P90. Male rl/+ mice vehicle treated show significantly less PCs in comparison to +/+ mice, $^{*}p < 0.001$; Male rl/+ mice 17β-E-treated show significantly less PCs in comparison to +/+ mice, $^{*}p < 0.001$).

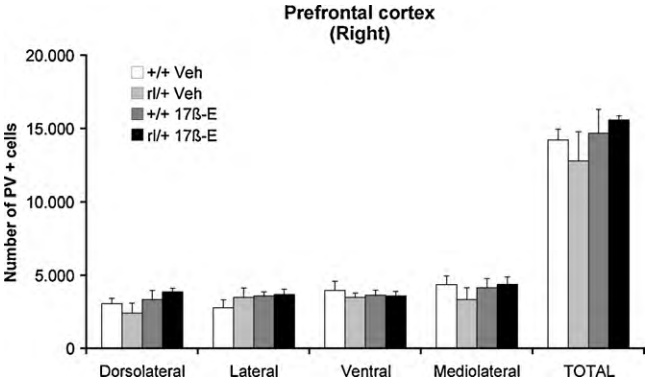

Figure 5 Stereological counts of PV+ GABAergic interneurons in coronal sections of the right prefrontal cortex of +/+ and rl/+ adult male mice in comparison to adult male mice administered with 17β-E. Similar results were obtained in the left prefrontal cortex (see text).

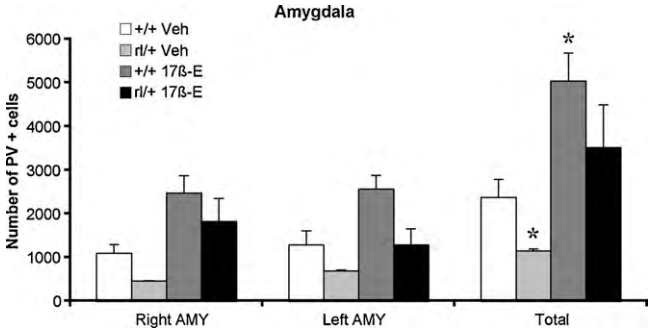

Figure 7 Stereological counts of PV+ GABAergic interneurons in coronal sections of the amygdala of +/+ and rl/+ adult male mice in comparison to adult male mice administered with 17β-E. PV+ cells were counted separately in the right and left amygdala. Total numbers represent the sum of left and right amygdalae. Genotype effect: rl/+ mice showed a significant reduction of total numbers of PV+ GABAergic interneurons in the amygdala. Treatment effect: The total number of PV+ cells is significantly increased in 17β-E-treated +/+ mice when compared to vehicle-treated +/+ mice. Notice that the numbers of PV+ neurons in 17β-E-treated rl/+ mice is not significantly different from vehicle-treated +/+ mice. $^{*}P < 0.05$, significantly different from vehicle-treated +/+ mice.

increased the number of PV+ GABAergic interneurons both in +/+ and in rl/+ mice (treatment, $F(1,12) = 6.1$, $p < 0.05$). Thus, such an increase normalized the numbers of GABAergic interneurons in rl/+ individuals compared to vehicle-injected +/+ subjects. However, 17β-E- increased the number of PV+ interneurons above those recorded in +/+ controls (see Figs. 6 and 7). Symbols (asterisks) in Fig. 7 indicate the difference

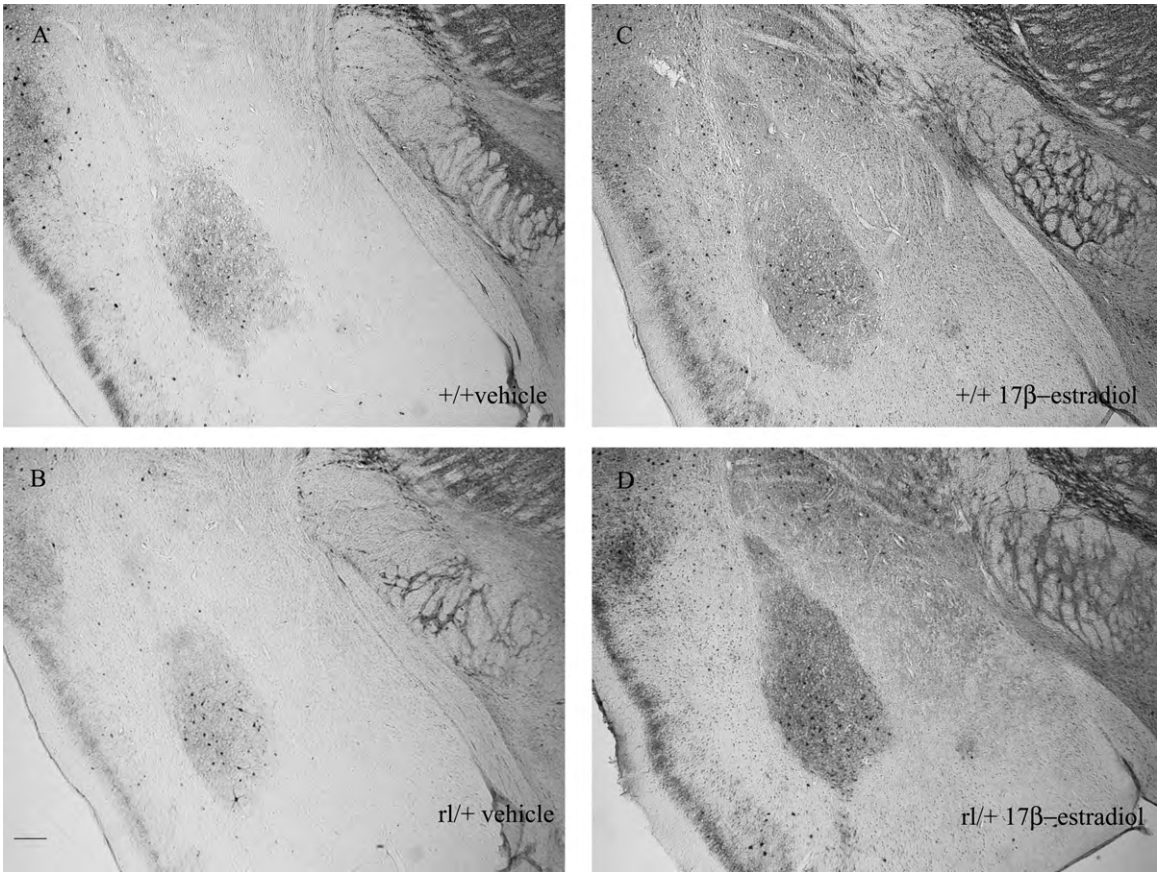

Figure 6 Representative photomicrographs of coronal sections through the amygdala of +/+ and rl/+ male mice at P90, immunostained for Parvalbumin (PV+). (A and B) PV+ of both genotypes under control conditions (vehicle); (C and D) PV+ of both genotypes after induction with 17β-Estradiol. Scale bar 100 mm.

between the target group in comparison with the +/+-veh group (white bar). Separate graphs for the left- and right-side of the amygdala are reported for the sake of completeness.

4. Discussion

Here, we first investigated whether rl/+ mice display behavioral and neuroanatomical abnormalities isomorphic to symptoms frequently observed in ASD patients, and then whether a neonatal estradiol treatment can compensate for these observed alterations. In line with our predictions, we observed that, in the absence of major disturbances in adult motor coordination (rotarod) and in fear-related responses, (i) infant rl/+ mice show reduced motivation to reach the nest-cage odor scented area compared to +/+ subjects; (ii) adult rl/+ mice display increased perseverative responding in an attentional set-shifting. These alterations were paralleled, in adulthood, by a decrease in the number of GABAergic inhibitory neurons in the cerebellum (Purkinje cells) and in the amygdala (parvalbumin-positive interneurons). Treatment with 17β-E was effective in mitigating such differential phenotype. However, in partial contrast with our previous observations — showing a compensatory effect of neonatal estradiol administration on cerebellar abnormalities at P15 (Biamonte et al., 2009) — neonatal 17β-E administration did not result in increased PCs number in adult rl/+ mice.

Finally, estradiol treatment *per se* had significant effects in +/+ subjects whereby it (i) significantly reduced bodyweight throughout development; (ii) reduced the capabilities to reach the goal area in the homing test in infancy measured as the percentage of subjects reaching the nest-scented portion of the apparatus; (iii) deteriorated reversal learning abilities and increased the number of amygdaloid GABAergic interneurons in adulthood.

4.1. rl/+ mice display behavioral alterations isomorphic to ASD

To further demonstrate that rl/+ mice might constitute a valid ASD experimental model, we addressed individual responses of wild-type and rl/+ mice to experimental tasks assessing behavioral domains typically impaired in children with ASD (Shu et al., 2001; Crawley, 2007).

Accordingly, rl/+ mice (i) were impaired in the motivation to reach the home-cage scented area on P9 and (ii) performed an increased number of perseverative responses in the reversal learning of an attentional set-shifting task in adulthood. Homing test is considered a valid indicator of neonatal social preference in mice (Alleva et al., 1985; Alleva and Bignami, 1986), whereby it 'requires adequate [...] associative and discriminative capabilities that allow the pup to become imprinted by the mother's odor, to remember it, and to recognize it among others' (Bignami, 1996). Thus, impaired homing performance has been associated with the reciprocal interaction deficits observed in autistic patients early in infancy (Rutgers et al., 2004; Laviola et al., 2009). Importantly these effects were apparently independent of general locomotion and neurobehavioral development (Alleva et al., 1985; Laviola et al., 2006), which were similar in all groups. Yet, social deficits did not persist into adulthood. Specifically, in contrast with our predictions, social interaction levels in adult rl/+ mice were apparently indistinguishable from those exhibited by +/+ individuals. Since Podhorna and Didriksen (2004) failed to observe major social interaction deficits in adult rl/+ male mice in a classical social interaction test, we adopted a task that is specifically designed for ASD-like behavioral abnormalities (Crawley, 2004, 2007). Notwithstanding major methodological differences, ranging from stimulus subject selection (white NMRI mice in Podhorna and Didriksen, 2004 and C57 wild types in this study) to the accessibility of the social stimulus (complete physical interaction in Podhorna and Didriksen, 2004 vs. sniffing in the current study), these data converge to indicate that social deficits in adult rl/+ mice are not prominent. Although present data indicate that adult rl/+ mice may not represent a valid mouse model for social deficits in adulthood, alternative testing strategies may further clarify this aspect in the future. For example, in order to dissociate basal from chemically challenged levels of social interaction experimental subjects may be challenged with exposure to psychoactive drugs (Podhorna and Didriksen, 2004).

Adult rl/+ mice committed a higher number of errors (perseverative responding) before the completion of a reversal-learning task, analogous to the human Wisconsin Card Sorting Task (WCST). Accordingly, South et al. (2007) observed a significant correlation between repetitive behaviors and WCST performance in ASD patients. This test, (Birrell and Brown, 2000; Colacicco et al., 2002; Garner et al., 2006), allows distinguishing between simple discriminatory aspects (simple and compound discriminations) and more complex cognitive processes (Garner et al., 2006). In accordance with previous studies (Brigman et al., 2006), rl/+ mice showed a selective impairment in reversal learning of the compound discrimination. These results are in partial contrast with previous studies investigating cognitive abilities in rl/+ mice. Specifically, Krueger et al. (2006) failed to observe attentional deficits in rl/+ mice in a three-choice serial reaction time task in an operant conditioning chamber. Likewise, Salinger et al. (2003) did not observe major differences between in rl/+ and +/+ mice in a thorough test battery addressing social, emotional, locomotor and cognitive performances. However, major methodological discrepancies may partly explain the different results obtained in the present study and in previous ones. Specifically, the attentional set-shifting task used in the present study presents some major advantages compared to previous ones (see Garner et al., 2006 for a discussion). Briefly, the use of ethologically salient conditioning stimuli (medium texture and odors) allows mice to display species-typical behaviors like digging and directing the attention towards odor cues. As also discussed by Garner et al. (2006), these aspects, coupled with the possibility to use internal controls for the different stages of the task, may result in an increased sensitivity of this task compared to previous ones.

In order to address the specificity of these alterations and exclude the possibility that they were due to major alterations in motor activity and neurobehavioral development, we also evaluated the neonatal grasping reflex and levels of general locomotion in infancy and adulthood. Additionally, in order to exclude major deficits in motor coordination we also directly assessed rotarod performance. These tests failed to show any difference between rl/+ and control subjects.

4.2. Compensatory effects of neonatal estradiol on behavioral deficits in infant rl/+ mice

Control rl/+ mice showed a decreased performance in the homing test, while estradiol administration on P5 compensated for this deficit. One caveat to these findings is that a majority of pups did not reach the target area. Though simple, the homing test addresses sensorimotor learning in neonate mice. In particular, it requires the integration of olfactory cues and locomotion, a form of sensorimotor integration. While the cerebellar role in olfaction is a subject of debate (see e.g. Kronenbuerger et al., 2010), the cerebellar cortex has been hypothesized to guide the development of sensorimotor maps in premotor networks, and PCs play a crucial role in this model (Hua and Houk, 1997); hence, a reduced number of PCs could interfere with developmental integration of olfactory and locomotor maps. Additionally, we previously observed that estradiol administration on P5 induced a short-term compensation of the cerebellar abnormalities (see Biamonte et al., 2009). Importantly, estradiol influences on nest homing (this study) and on PCs (Biamonte et al., 2009) occur during the same neonatal time window, supporting a role of cerebellum in the homing test. Alternatively, it could be proposed that the altered homing performance and the compensatory effects of estradiol were mediated by extracerebellar circuits, like the amygdala (see below). Identifying whether one or the other hypothesis will come to prevail will require additional independent studies aimed at investigating the short-term consequences of estradiol administration on amygdalar abnormalities.

Alternatively, baseline olfactory deficits in rl/+ mice *per se* may partly explain the observed results (see Larson et al., 2003). However, in this study we did not observe significant between-group differences in odor processing in adult mice (see below). Additionally, the hypothesis that reelin-dependent olfactory deficits explain the differences observed in homing performance would be hardly reconciled with the observation that +/+ estradiol-treated mice did show an analogous deficit.

4.3. Compensatory effects of neonatal estradiol on behavioral deficits in adult rl/+ mice

Neonatal estradiol treatment had long-term behavioral consequences in adulthood, ameliorating the performance of rl/+ in the set-shifting task to levels comparable to wild-type mice. While there is abundant evidence of the role of endogenous estrogens, and beneficial effects of long-term estrogen treatment, on cognitive function, it is perhaps surprising that a single dose of estradiol, administered in neonatal life, had such lasting effects on adult cognition. However, we believe that a transient correction of PC dysfunction during a critical period of differentiation and synaptogenesis of PCs may benefit the adult phenotype.

While a direct role of the cerebellum in the set-shifting task has yet to be demonstrated, cerebellar abnormalities have been associated with special deficits in attention in humans and primates (Schmahmann, 2004; Steinlin, 2008; Strick et al., 2009). Since adult rl/+ mice show reduced numbers of PCs compared to +/+, independently of treatment, present data indicate that the rescue effect of estradiol on neonatal PCs does not persist until adulthood. In contrast to the findings in the cerebellum, we observed that amygdala alterations in adult rl/+ mice are neutralized by neonatal estradiol administration. As already proposed above, these data suggest that the neonatal compensatory effect at the level of cerebellum may be translated to other brain areas that are functionally connected to the cerebellum, and persist into adulthood. It remains to be seen whether estradiol has a compensatory effect in the amygdala also when administered in adult life.

4.4. Relationship between behavioral deficits and neuroanatomical alterations

Although cerebellar dysfunctions may directly relate to cognitive impairments, they may also exert negative effects on cognitive performance through a dysfunctional cerebro-cerebellar loop, involving the prefrontal cortex (Leggio et al., 2008). Specifically, the cerebellum has been proposed to serve as an integrative area providing 'correct predictions about the relationship between sensory stimuli' (Timmann et al., in press). In order to investigate whether the prefrontal component of the cerebro-cerebellar loop was also impaired in rl/+ subjects, we evaluated the number of parvalbumin-positive GABAergic interneurons in the prefrontal cortex. Fast-spiking, PV+ GABAergic interneurons in the mammalian cortex are thought to play a crucial role in attentional processes (Sohal et al., 2009). In contrast with our predictions, mice of the different groups showed an indistinguishable anatomical phenotype in the prefrontal cortex. Conversely, adult +/+ and rl/+ mice showed marked basal differences in the number of PV+ GABAergic interneurons in the amygdala, and a differential regulation following estradiol exposure. Specifically, estradiol compensated for the interneuron reduction observed in rl/+ mice. In addition to the cerebellar abnormalities, the differential regulation of the amygdala may partially explain the cognitive performance observed in the attentional set-shifting task. According to recent experimental and theoretical work, this structure is implicated in affective labeling of external sensory stimuli, goal-directed behavior, and planning and decision making (e.g. Mohanty et al., 2008; Mirolli et al., in press). In humans, the amygdala mediates cognitive performance in motivation-dependent attentional set-shifting paradigms (Mohanty et al., 2008), and shows a specific hemodynamic activation during an attentional set-shifting task in response to food-related visual cues (Mohanty et al., 2008). The rodent version of the attentional set-shifting task utilizes a food reward in food-restricted animals, i.e. a highly relevant stimulus. In the light of the numerous tactile and olfactory inputs it receives, the rodent amygdala may thus be primarily responsible for determining the motivational value in this task. Interestingly, lesions of the amygdala, but not of the hippocampus, indicate that the amygdala is involved in working memory for the reward value of a stimulus (Gilbert and Kesner, 2002). Thus, amygdala involvement in the working memory component of the set-shifting task may partly explain both the baseline difference between wild-type and rl/+ individuals and the observed compensatory effects of estradiol. Furthermore, our observations nicely fit with the view that amygdala and

cerebellum may constitute a functional unit, although their functional interconnections have yet to be demonstrated (Strick et al., 2009).

Whilst central processing of the stimulus salience may explain the observed attentional performance, an alternative explanation might involve more peripheral components of the task. Specifically, since rl/+ mice have been reported to show olfactory deficits (Larson et al., 2003), and the task adopted in this study involves a large olfactory component, it could be argued that peripheral processing of the scents used in the task may explain the observed responses. However, the olfactory component is assessed during the early stages (SD) of the task, whereby the baited bowl is recognized upon odor cues. Since SD was similar among experimental groups, either olfaction was not altered in rl/+ mice or the deficit was subtle enough not to affect the performance in this task.

Yet, it still remains unanswered why amygdalar morphological alterations dissociate from the responses observed in fear conditioning. Specifically, we expected rl/+ mice to display impaired fear conditioning in light of the reduced number of GABAergic interneurons in the amygdala. Although our results do not contrast this hypothesis, neither do they support it. We believe that the lack of effect may be due to limited sensitivity of the test adopted (i.e. floor effect), or to the fact that the neuronal alterations we observed were selective enough to affect attentional shifting, without resulting in an overt fear-conditioning deficit. Literature data on anxiety related measures in rl/+ mice are however conflicting. For example, whereas Podhorna and Didriksen (2004) observed no significant differences in the light—dark test, Salinger et al. (2003) observed a differential approach-latency to an unknown object in rl/+ compared to +/+ controls. Finally, we recently observed that rl/+ mice spent a significantly higher amount of time in the open sectors of an elevated plus maze compared to +/+ subjects (Ognibene et al., 2007) thus suggesting reduced anxiety. Future studies will be aimed at clarifying whether differences in amygdalar regulations relate to stable differences in emotional processing.

4.5. Differential effects of neonatal estradiol in rl/+ and +/+ mice

Excess estradiol in 'normal' +/+ infant mice reduced the percentage of subjects reaching the goal area in the homing test and had no effect on grasping reflex and USV emission. Additionally, it reduced body-weight gain, deteriorated cognitive performance and increased time spent in proximity of the unfamiliar individual in the social interaction test. Furthermore, estradiol administration in +/+ subjects resulted in an up-regulation of amygdalar interneurons, and a poorer performance in the set-shifting task. While this finding seems counterintuitive, it follows the same trend we observed in our previous paper (Biamonte et al., 2009) regarding the effect of estradiol on reelin mRNA. Indeed, we observed that estradiol led to a normalization of reelin mRNA levels in rl/+ mice, while reelin mRNA levels were increased above control levels in +/+ mice. This may explain the opposite effects of estrogen treatment in +/+ vs. rl/+ genotypes: in rl/+ mice estradiol may normalize an altered ratio of excitatory/inhibitory synapses, while in +/+ animals estrogen may alter a previously normal excitation/inhibition ratio.

Taken together, our observations suggest an important interplay between reelin and estradiol in fine-tuning the number of GABAergic neurons in various neural structures. A related crucial question concerns the mechanism of modulation of PV+ GABAergic interneurons in the amygdala by estrogen administration. Although it is thus far not established that increased reelin expression by itself, or in conjuction with increased estrogen levels, is causally related to increased Purkinje cell survival, it is tempting to speculate that a similar mechanism may operate in GABAergic interneurons in the amygdala.

4.6. Implications for the neurobiology of ASD

Our findings confirm that reduced reelin expression in mice is associated with neuroanatomical alterations, in particular with a small but significant loss of PCs in the cerebellum, and with neurobehavioral abnormalities. Interestingly, Maloku et al. (2010) have also found a similar PC loss in adult reelin-haploinsufficient mice, and have interpreted their findings in the context of reelin deficiency and cerebellar abnormalities observed in schizophrenia, thus underscoring the links between reelin and psychosis. The question why reelin haploinsufficiency leads to cell loss in the male but not in the female cerebellum, and how high levels of estradiol at P5 inhibit cell loss, cannot be answered yet, but recent data indicate that PCs show a peak of apoptosis during the first postnatal week in the mouse (Jankowski et al., 2009). It is tempting to speculate that PC loss in rl/+ mice is due to apoptosis, and that estradiol inhibits cell death by increasing reelin expression. Recent genetic studies have highlighted a link between variants of the reelin gene and risk of psychosis in women (Shifman et al., 2008; Goes et al., 2009). In conclusion, there is accumulating evidence that an interaction between altered reelin function and altered estrogen/androgen balance could be an important pathophysiological mechanism in ASD, as well as other psychoses. Furthermore, the present results call for a closer focus on the amygdala and its functional connections with the cerebellum. These connections could be a critical target where reelin downregulation and hormone levels could converge to generate social and cognitive deficits.

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Conflict of interest

The authors have no conflict of interest to disclose.

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